Proprotein Convertase Subtilisin/Kexin Type 9 Antagonism Reduces Low-Density Lipoprotein Cholesterol in Statin-Treated Hypercholesterolemic Nonhuman Primates

Hong Liang, Javier Chaparro-Riggers, Pavel Strop, Tao Geng, Janette E. Sutton, Daniel Tsai, Lanfang Bai, Yasmina Abdiche, Jeanette Dilley, Jessica Yu, Si Wu, S. Michael Chin, Nicole A. Lee, Andrea Rossi, John C. Lin, Arvind Rajpal, Jaume Pons, and David L. Shelton

Rinat Laboratories, Pfizer Inc., South San Francisco, California

Received August 29, 2011; accepted October 11, 2011

ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) promotes the degradation of low-density lipoprotein (LDL) receptor (LDLR) and thereby increases serum LDL-cholesterol (LDL-C). We have developed a humanized monoclonal antibody that recognizes the LDLR binding domain of PCSK9. This antibody, J16, and its precursor mouse antibody, J10, potently inhibit PCSK9 binding to the LDLR extracellular domain and PCSK9-mediated down-regulation of LDLR in vitro. In vivo, J10 effectively reduces serum cholesterol in C57BL/6 mice fed normal chow. J16 reduces LDL-C in healthy and diet-induced hypercholesterolemic cynomologous monkeys, but does not significantly affect high-density lipoprotein-cholesterol. Furthermore, J16 greatly lowered LDL-C in hypercholesterolemic monkeys treated with the HMG-CoA reductase inhibitor simvastatin. Our data demonstrate that anti-PCSK9 antibody is a promising LDL-C-lowering agent that is both efficacious and potentially additive to current therapies.

Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) has emerged in recent years as a promising therapeutic target for lowering low-density lipoprotein-cholesterol (LDL-C). In humans, gain- and loss-of-function PCS9 variants associate with hypercholesterolemia or hypocholesterolemia, respectively (Abifadel et al., 2009). These mutations have been found to reduce or increase cardiovascular heart disease risk. Furthermore, humans who are null for PCSK9 seem to be healthy and normal (Horton et al., 2007; Abifadel et al., 2009).

PCSK9 is a secreted serine protease that is made primarily by the liver and intestine. It consists of a signal peptide, a prodomain, a catalytic domain, and the histidine-rich C-terminal domain. The prodomain is self-cleaved in the endoplasmic reticulum after synthesis and covalently bound to the mature protein thereafter. PCSK9 binds to the epidermal growth factor-like repeat A (EGF-A) domain on LDLR in a calcium-dependent manner (Zhang et al., 2007). X-ray crystallography studies of cocrystals show that a region of the PCSK9 catalytic domain, distinct from the actual catalytic site, makes direct contact with the EGF-A domain (Kwon et al., 2008). Data show that PCSK9 asserts its function primarily through binding to hepatocyte LDLR and preventing LDLR recycling to the cell surface after endocytosis. This results in reduced LDLR levels, decreased cellular uptake of LDL-C, and higher LDL-C levels in blood (reviewed in Horton et al., 2009). Some evidence suggests that this occurs primarily on the cell surface of hepatocytes, after PCSK9 has been secreted. However, it has been suggested that intracellular PCSK9 may target LDLR for degradation before it is chaperoned to the cell surface (Poirier et al., 2009), providing an additional mechanism for reducing LDLR levels. Thus the site of action of PCSK9 in vivo remains an open question.

This work was supported by Pfizer Inc.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

http://dx.doi.org/10.1124/jpet.111.187419.

The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: PCSK9, proprotein convertase subtilisin/kexin type 9; PCSK9(−/−), PCSK9 knockout; huPCSK9, human PCSK9; LDL, low-density lipoprotein; LDLR, LDL receptor; LDL-C, LDL-cholesterol; ECD, extracellular domain; HDL, high-density lipoprotein; HDL-C, HDL-cholesterol; HC, high cholesterol; HF, high fat; TC, total cholesterol; EGF-A, epidermal growth factor-like repeat A; NHP, nonhuman primate; ELISA, enzyme-linked immunosorbent assay.
Elimination or reduction of PCSK9 activity via genetic deletion or pharmaceutical intervention, such as antisense, RNA interference, or monoclonal antibodies, has been shown to cause an increase in hepatocyte LDLR and a subsequent reduction in serum LDL-C levels (Rashid et al., 2005; Graham et al., 2007; Frank-Kamenetsky et al., 2008; Chan et al., 2009; Gupta et al., 2010; Ni et al., 2011). Some of these interventions have also been shown to effectively reduce LDL-C and serum lipid levels in nonhuman primates (NHPs) with normal diet.

Statins are currently the most successful and widely used class of therapy for hyperlipidemia. Through inhibiting HMG-CoA reductase and cholesterol synthesis, they activate the transcription factor sterol response element binding protein 2 and subsequently induce LDLR expression. Activated sterol response element binding protein 2 also induces the expression of other genes involved in cholesterol metabolism, including PCSK9. In addition, statins have been found to up-regulate hepatocyte nuclear factor 1α, which induces PCSK9 expression (Dong et al., 2010). These increased levels of PCSK9, in turn, increase the degradation of LDLR protein, thus probably dampening the full potential effect of statin treatment (Dubuc et al., 2004; Park et al., 2004). It is therefore speculated that statins and PCSK9 inhibitors may have an additive or even synergistic effect on LDL-C levels. Indeed, mice lacking PCSK9 have been shown to be hypersensitive to statins (Rashid et al., 2005). Epidemiological studies have shown increased PCSK9 levels upon statin use and correlated statin responsiveness to PCSK9 loss-of-function mutants (Berge et al., 2006; Thompson et al., 2009; Konrad et al., 2011). Reports also indicated other lipid-lowering therapies, such as fenofibrate and the Niemann-Pick C1-Like 1 inhibitor ezetimibe, can increase circulating PCSK9 protein levels (Konrad et al., 2011). Short interfering RNA-mediated knockdown of Pseks9 led to a greater reduction in serum non-HDL-C in mice in combination with ezetimibe (Ason et al., 2011), suggesting that a PCSK9 inhibitor may have an additive effect with these major classes of current lipid-lowering medications as well. Population-based studies and pivotal clinical trials have shown that for most people there is a clear heart health benefit of lowering blood LDL-C to levels possibly even lower than the current Adult Treatment Plan III guidelines (reviewed in O’Keefe et al., 2004). Current therapies, although well tolerated and efficacious in most incidences, are insufficient to provide optimal lowering of LDL-C in a subset of patients because of inadequate efficacy, tolerability, or safety (reviewed in Costet, 2010). Cardiovascular heart disease is still the number one cause of death in the developed world, even with the advent and wide adoption of current lipid-lowering therapies. There is clearly a need for new additive or replacement therapies that are effective and safe.

We have identified a monoclonal antibody, J16, which binds to PCSK9 and neutralizes its effect on LDLR degradation. Structural studies on cocrystals of this antibody bound to PCSK9 show that it binds to the same site on PCSK9 as LDLR. This LDLR-binding site is evolutionally conserved among rodent, NHP, and human, making J16 a potent cross-species PCSK9 inhibitor. By using this antibody in mice and comparing its efficacy to that of genetic deletions we could directly test the hypothesis that little, if any, effect on LDLR is mediated by intracellular PCSK9 under normal physiological conditions. We show that J16 effectively reduces serum cholesterol in mice, to a level similar to that observed in PCSK9 knockout (PCSK9(−/−)) mice, demonstrating that the majority, if not all, of PCSK9 function in vivo is mediated via circulating PCSK9. We also show that J16 can selectively lower LDL-C in NHPs with normal or elevated cholesterol levels. Finally, we were able to test and confirm the hypothesis that PCSK9 antagonism can provide further LDL-C lowering when coadministered with a statin.

Materials and Methods

Generation of Proteins and Antibodies. Recombinant human PCSK9 protein was produced as described previously (Cunningham et al., 2007). Mouse and cynomolgus PCSK9 protein was produced by cloning the cDNA sequences into mammalian expression vector PRK5 with the addition of a six-His tag at the C terminus, transiently transfected and expressed in human embryonic kidney 293 cells using Lipofectamine (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Recombinant proteins were purified from conditioned media by using a Ni column (QiAGEN, Valencia, CA) and standard techniques. LDLR extracellular domain (ECD) was purchased from R&D Systems (Minneapolis, MN).

Mouse antibodies to human PCSK9 were generated by immunizing wild-type BALB/c and 129/BL6 PCSK9(−/−) mice (Rashid et al., 2005) with recombinant human PCSK9 (huPCSK9). Immortalized lymphocytes were generated from spleen by fusion with an established cell line by using standard hybridoma technology. Clones were screened for the ability to bind human PCSK9 by ELISA and purified from hybridoma cultures by using monoclonal antibody select beads (Thermo Fisher Scientific, Waltham, MA) and further screened for effects on huPCSK9-mediated LDLR down-regulation in Hep7 cells by using Western blot analysis (see below).

Mouse antibody J10 was humanized and affinity-matured to antibody J16 by using standard humanization and affinity maturation strategies (Bostrom et al., 2009).

Animal Studies. C57BL/6 mice were purchased from Charles River Breeding Laboratories (Portage, MI); LDLR(−/−) and LDLR(+/−) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were acclimated to our facility for 2 weeks before the start of experiments. PCSK9(−/−) mice (Rashid et al., 2005) were purchased from The Jackson Laboratory and bred at our facility. Animals were housed conventionally under ambient conditions, with free access to water and standard rodent chow unless otherwise specified. Diets of high fat (HF; 60% kcal fat, 20% kcal protein, and 20% kcal carbohydrate) and HF/high cholesterol (HC; 20% kcal protein, 40% kcal carbohydrate, and 2.8 mg/kcal cholesterol) were purchased from Research Diets (New Brunswick, NJ). Mice were fasted for 4 h before serum samples were collected.

A total of 16 male adult cynomolgus macaques (Macaca fascicularis), ranging from 5 to 9 years of age and 4 to 6 kg of weight, were used for the dose response of J16. Animals were randomized based on LDL-C levels at day −7 and weight (n = 4/group). Animals were housed in stainless-steel individual cages and fed ad libitum with Harlan (Indianapolis, IN) Primate Diet 2050 and water, and fasted overnight before plasma samples were collected.

Twelve female adult cynomolgus macaques (M. fascicularis), ranging from 3.5 to 4 kg of weight, that were fed a primate high-fat diet (62.1% kcal fat, 21.7% kcal carbohydrate, 16.2% kcal protein, and 0.1 mg/kcal cholesterol) (Harlan Primate Diet TD.06278) for at least 18 months were used in this study. Animals were housed in individual cages and maintained on the high-fat diet with ad libitum access to water. Animals were randomized into three groups based on LDL-C levels at day −7 and weight (n = 4/group). Groups 2 and 3 were administered via oral gavages with rosuvastatin calcium (Crestor; AstraZeneca Pharmaceuticals LP, Wilmington, DE) during study days 1 to 55 as a whole tablet at 10 mg/animal and 10 mg/kg on days...
63 to 76 as a fine suspension in a 5% Gum Arabic vehicle. Simvastatin (Zocor; DuPont Merck Pharmaceutical Co., Wilmington, DE) administered to the animals on study days 84 to 133 as a fine suspension in a 5% Gum Arabic vehicle at 50 mg/kg. J16 (3 mg/kg) was administered via intravenous bolus injection in group 1 and 2 animals on day 14 and 3 groups of animals on day 105. Fasted plasma samples were taken at the indicated time points from days 7 to 133. Rosuvastatin calcium and simvastatin were purchased at a local pharmacy. All animal maintenance and handling were conducted in accordance with our Institutional Animal Care and Use Committees in Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facilities.

Serum and plasma samples were analyzed for lipid measurements (total cholesterol, HDL-C, LDL-C, and triglyceride) by using the Ace Alera Clinical Chemistry System (Alfa Wassermann, West Caldwell, NJ). Results of lipid measurements were analyzed and graphed by using Prism software (GraphPad Software Inc., San Diego, CA).

**Results**

**Identification of Antagonistic Anti-PCSK9 Antibodies.** We screened for anti-PCSK9 monoclonal antibodies among hybridoma cell lines generated by fusing the spleen of various wild-type and PCSK9(-/-) mice immunized with recombinant huPCSK9 protein. More than 800 antibodies that were positive for PCSK9 binding by ELISA were tested for their ability to block recombinant huPCSK9-mediated LDLR degradation in Huh7 cells. Approximately 60 clones (7%) showed some degree of recovery of LDLR protein levels in the presence of PCSK9. Only four antibodies produced by hybridoma cell lines showed complete block of PCSK9 activity. It is noteworthy that all of these were obtained from PCSK9(-/-) mice and all were also cross-reactive to mouse PCSK9. These four could mutually block each other’s binding to PCSK9 in ELISA and biosensor assays, indicating that they share overlapping epitopes. One of them, J10, binds to recombinant human, cynomolgus monkey, and mouse PCSK9 with approximate affinities of 0.3, 0.5, and 2.7 nM, respectively, as determined by biosensor binding assays (Supplemental Fig. 1). Figure 1A shows J10 was able to completely rescue LDLR levels in Huh7 cells treated with exogenous mouse and human PCSK9. J10 also completely and dose-dependently blocked binding of 1.4 nM human PCSK9 to immobilized human LDLR ECD with an IC50 of 1 nM (Fig. 1B).

We engineered J10 into a human IgG2deltaA (Armour et al., 2002) and κ chain antibody and further improved its antigen binding affinity. The resulting antibody, J16, contains fully human sequence outside of the complementarity determining regions. It binds to recombinant human PCSK9 molecules with a KD of approximately 5 pM as determined by KinExA (Supplemental Fig. 2), to cynomolgus monkey with KD of less than 100 pM, and to mouse PCSK9 with KD of 35 pM, as determined by biosensor binding assays (Supplemental Fig. 1). J16 completely blocks human and mouse PCSK9 binding to immobilized LDLR ECD and can fully reverse the PCSK9-mediated LDLR suppression in Huh7 cells.

**Affinity Determination of Antibodies.** The binding kinetics and affinities of PCSK9 antibodies, J10 and J16, to recombinant human, cynomolgus monkey, and mouse PCSK9 were measured on a surface plasmon resonance Biacore 2000 biosensor (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The binding affinity of J16 to recombinant human PCSK9 was also determined by using a KinExA 3000 system (Sapidyne Instruments Inc., Boise, ID). Detailed methods are provided in the supporting information.
Epitope Determination of J16. The cocrystal structure of the Fab region of J16 with recombinant human PCSK9 revealed that J16 binds mainly to the catalytic domain of PCSK9 and partly to the C terminus of the prodomain (Fig. 2A). J16 binds to a nonlinear, three-dimensional epitope that almost perfectly overlaps with the LDLR EGF-A domain binding site on PCSK9 (Kwon et al., 2008) (Fig. 2B). The interaction between PCSK9 and J16 is mediated by both light and heavy chains, with the main contribution coming from heavy-chain complementarity determining region 3. The surface area covered by J16 is approximately 788 Å², whereas the LDLR EGF-A domain buries 456 Å². A crystallographic data table and methods can be found in the supporting information.

Anti-PCSK9 Antibodies Reduce Cholesterol in Mice and Monkeys. The ability of the mouse antibody J10 to modulate blood cholesterol levels in vivo was tested in mice. Because rodents carry LDLR binding lipoprotein in their HDL particles, the increase of LDLR that would occur via PCSK9 inhibition is expected to cause a decrease in LDL-C, HDL-C, and total cholesterol in the rodent, as was seen in the PCSK9(-/-) mice (Rashid et al., 2005). Because of the very low levels of LDL-C in wild-type mice, which are difficult to reliably measure and calculate, we observed the most consistent and significant effect of PCSK9 neutralization in mice on total and HDL-C levels. When 10 mg/kg J10 was administered as a single intraperitoneal dose to C57BL/6 mice fed a normal diet, serum total cholesterol levels were maximally reduced to an average of 49.7 mg/dl at day 7 after treatment, a 46% reduction, compared with 92.5 mg/dl in saline-treated controls (Table 1). HDL-C level was on average 33.4 mg/dl in the J10-treated group compared with 58.4 mg/dl in saline-treated controls, representing a 43% reduction. There was no significant change in triglyceride levels.

Two sets of experiments were carried out to explore the mechanism of this effect on serum lipids. Liver LDLR levels were significantly increased (2.3-fold, p = 0.001) in mice 7 days after treatment with 10 mg/kg J10 (Supplemental Fig. 3). In addition, total cholesterol, HDL-C, and LDL-C levels in LDLR(-/-) mice were not affected by 10 mg/kg J10 treatment. J10 treatment was partially effective in LDLR(-/-) mice, with a 22% (27 mg/dl) reduction in total cholesterol, a modest but statistically significant 13% reduction in HDL-C, and a preferential 70% reduction in LDL-C (Table 1). These data are consistent with the effect of J10 being mediated by the inhibition of the effect of endogenous PCSK9 on LDLR.

![Fig. 1. Inhibition of PCSK9 function by J10 in vitro. A, Western blot analysis showing the effect of J10 on the ability of mouse and human recombinant PCSK9 to down-regulate LDLR protein levels in cultured HuH7 cells. B, the dose response of J10 (○) and an isotype control (▼) to block the binding of recombinant biotinylated huPCSK9 to immobilized recombinant LDLR ECD in vitro. Gray dotted line, signal with no biotinylated PCSK9 added. Black dotted line, signal with no antibody added. The average signals from duplicate samples are plotted.](image)

![Fig. 2. J16 binds to the same region of PCSK9 as the LDLR EGF-A domain. A, crystal structure of the PCSK9 bound to the J16 Fab. PCSK9 is shown in gray surface representation, and J16 Fab is shown in magenta (light chain) and blue (heavy chain) cartoon representation. B, view of epitope residues for J16 Fab and LDLR EGF-A on the PCSK9. PCSK9 is shown in gray surface representation. Residues buried upon J16 binding are shown in blue, and the outline of the buried surface for the PCSK9:LDLR complex is shown in red. Buried surface areas were calculated as described under Materials and Methods.](image)
To understand the differential effect of PCSK9 inhibition on HDL- and LDL-cholesterol in primates, we administered humanized J16 into cynomolgus monkeys fed a normal diet. J16 was administered as a single intravenous dose at 0.1, 1, 3, and 10 mg/kg (n = 4/group). J16 significantly and rapidly reduced LDL-cholesterol at all doses (Fig. 3A). The magnitude, duration, and accumulative percentage reduction over time calculated by area under the curve of the LDL-C lowering was dose-dependent (Fig. 3A; Supplemental Fig. 4). Administration of 0.1 mg/kg J16 caused a transient 50% drop in LDL-cholesterol levels at day 2 that quickly recovered by day 5. Dosing at 1 mg/kg reached a maximum effect of 71% reduction in LDL-cholesterol on day 5 and began to recover immediately thereafter, reaching predose levels by day 14. Dosing at 3 and 10 mg/kg maintained the 70% reduction in LDL-cholesterol levels until days 10 and 21 postdosing, respectively, and fully recovered by days 21 and 31, respectively. Total cholesterol levels were also significantly reduced in all dosing groups, with a dose response and time course similar to that of LDL-C and a maximum reduction seen in the 10 mg/kg group (up to 39%) lasting 21 days. There were no dose-related changes observed in HDL-cholesterol (Fig. 3B) or triglyceride levels throughout the study.

Effect of Anti-PCSK9 Antibodies in Hypercholesterolemic Models. We tested the effect of anti-PCSK9 antibodies on mice fed a HF diet (60% kcal fat) or a HF/HC diet (40% kcal fat with 2.8 mg/kcal cholesterol). Six-week-old C57BL/6 mice were fed HF or HF/HC diets for 6 weeks before the antibody treatments, which raised total cholesterol to 135 and 202 mg/dl, respectively, compared with the ~90 mg/dl we typically observe for animals fed normal chow. A single 10 mg/kg injection of J10 was given to mice fed a HF diet, and four consecutive weekly injections at 10 mg/kg were given to mice fed a HF/HC diet. To our surprise, neither treatment paradigm had a significant effect on serum total cholesterol, HDL-C, or LDL-C (Supplemental Tables S1 and S2). Furthermore, PCSK9(−/−) mice fed a high-fat/high-cholesterol diet also did not exhibit reduced cholesterol levels compared with those on normal chow in our hands (Supplemental Fig. 5). To further investigate the cause of the unresponsiveness of these diet-induced hypercholesterolemic mice to PCSK9 antagonism, we measured serum PCSK9 concentrations in these animals. Serum PCSK9 was reduced by 64% in mice fed a HF diet for 6 weeks (14.4 ± 1.6 ng/ml) compared with those on normal chow (40.4 ± 4.6 ng/ml) (n = 5/group).

We then investigated the efficacy of J16 in hypercholesterolemic nonhuman primates. Before the initiation of the study, the LDL-C levels of a cohort of cynomolgus monkeys were elevated to an average of 120 mg/dl, compared with the normal average levels of 50 mg/dl, by feeding them a diet containing 62% kcal fat and 0.1 mg/kcal cholesterol for more than 18 months. A single dose of 3 mg/kg J16 administered to these animals effectively lowered serum LDL-C levels by 64%.

### Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vehicle-Treated</th>
<th>J10-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC (mg/dl)</td>
<td>HDL-C (mg/dl)</td>
</tr>
<tr>
<td>Wild type</td>
<td>92.5 ± 2.5</td>
<td>58.4 ± 1.4</td>
</tr>
<tr>
<td>LDLR(−/−)</td>
<td>123.1 ± 1.7</td>
<td>70.2 ± 1.1</td>
</tr>
<tr>
<td>LDLR(−/−)</td>
<td>253.6 ± 11.2</td>
<td>77.8 ± 2.5</td>
</tr>
</tbody>
</table>

N.A., not available.

*P < 0.001 compared with vehicle-treated group.
by day 3 post-treatment; serum LDL-C levels gradually returned to predosing levels by approximately 2.5 to 3 weeks post-treatment (Fig. 4A). The percentage of reduction and the duration of the effect were very similar to that observed in monkeys fed normal chow and treated with the same dose (3 mg/kg) of J16 (Fig. 3A). HDL-C levels were not affected by the treatment (Fig. 4B). Total cholesterol followed a similar time course as LDL-C, with maximum lowering of 41% (Fig. 4C). There were no significant changes related to J16 treatment in fasting triglyceride levels throughout the study.

**J16 Reduces LDL-C in Statin-Treated Hypercholesterolemic Monkeys.** To test the pharmacodynamic interactions between J16 and HMG-CoA reductase-inhibiting statins, we administered rosuvastatin calcium or simvastatin daily to hypercholesterolemic monkeys. We were surprised to find that no effect was observed on the serum levels of total cholesterol or LDL-C after the daily administration of a low dose (10 mg/animal) of rosuvastatin calcium for 6 weeks or a subsequent daily administration of a high dose (20 mg/kg) for 2 weeks. Liquid chromatography-mass spectrometry was used to measure plasma rosuvastatin concentrations (Martin et al., 2002). Rosuvastatin (4 ± 4 and 23 ± 12 ng/ml) was detected in plasma samples taken on day 10 of low or high rosuvastatin calcium dosing, respectively, confirming proper drug exposure in these animals. Upon switching the animals to daily administration of a high dose (50 mg/kg) of simvastatin, their LDL-C levels reached a maximal reduction of 43% at day 5 and stabilized thereafter (Fig. 4D). After 3 weeks of 50 mg/kg/day simvastatin administration, these animals were treated with vehicle or a single dose of 3 mg/kg J16 while still receiving 50 mg/kg/day simvastatin. J16 dosing caused an additional 65% reduction in LDL-C by day 5 and returned to predosing levels within 2 weeks (Fig. 4D), demonstrating that the PCSK9 antagonist can greatly reduce LDL-C levels on top of a statin. Daily simvastatin treatment caused a trend of gradual reduction in HDL-C. Treatment of J16 had no significant effect on HDL-C levels (Fig. 4E). Total cholesterol levels gradually reduced after daily simvastatin treatment, reaching a maximal 42% reduction at day 14. Single injection of J16 produced an additional 36% maximal lowering 5 days after dosing (Fig. 4F). The animals were closely monitored throughout the duration of the study for clinical signs, body weight, food intake, hematology, and serum chemistry. No meaningful changes related to rosuvastatin calcium, simvastatin, or J16 treatment were observed. More specifically, liver function, as judged by serum alanine aminotransferase and aspartate aminotransferase levels, and kidney function, as judged by serum creatinine, urea, and electrolytes levels, were not altered by statin or J16 treatment.

**Discussion**

Since the discovery of its role in cholesterol metabolism and LDLR regulation and the realization that PCSK9 is a promising therapeutic target for LDL-C lowering, the domains and structures important for PCSK9 function have been under intense investigation. The protease activity has been shown to be necessary for self-cleavage, folding, and secretion of the mature PCSK9 protein, but it is not involved in LDLR degradation (Li et al., 2007; McNutt et al., 2007). The binding epitope of PCSK9 to EGF-A of the LDLR has been localized to a conserved patch on the catalytic domain (Cameron et al., 2008; Kwon et al., 2008). The prodomain and the C terminus have also been implicated in PCSK9 activity or even direct binding to LDLR (Kwon et al., 2008; Mayer et al., 2007).
We have searched for complete blockers for PCSK9 among more than 800 ELISA-positive antibodies. Although approximately 7% of antibodies showed some blocking activity, potent blockers that can fully inhibit PCSK9 function were derived only from immunizations in a PCSK9(-/-) mouse. One of those antibodies was humanized and affinity-matured, and the X-ray structure of the Fab/PCSK9 complex shows the antibody binds to a three-dimensional epitope that is nearly identical to that of the EGF-A domain of LDLR. These results suggest that the LDLR binding epitope is critical in fully antagonizing PCSK9 function. This epitope is evolutionarily conserved from invertebrate to vertebrate (Cameron et al., 2008), hence it may be difficult to raise antibodies against it in wild-type mice. In fact, the residues of the J16 epitope on PCSK9 are 100% identical between mouse and human PCSK9, compared with the 78% overall identity and the 69% identity of the remaining surface residues. Our experience demonstrates the power of using knockout mice in generating functional antibodies. Consistent with our observations, monoclonal antibodies that have been reported to have potent PCSK9 blocking activities sterically block the binding of LDLR (Chan et al., 2009; Duff et al., 2009; Ni et al., 2011). One of these antibodies was generated by screening of human libraries; another was generated in mice engineered to express fully human antibodies, suggesting the immune tolerance and repertoire of these “humanized” mice are distinct from that of a wild-type mouse.

The critical cellular location where PCSK9 asserts its function under physiological conditions is still not clearly established. Many studies have demonstrated an important role for secreted PCSK9 in LDLR down-regulation. In vitro, recombinant PCSK9 binds to LDLR ECD (Cunningham et al., 2007), and exogenous PCSK9 protein effectively reduces both LDLR protein levels and LDL uptake in cultured cells (Lalanne et al., 2005; Maxwell et al., 2005). In vivo, injection or infusion of recombinant PCSK9 or blood from paired parabiotic mice overexpressing PCSK9 as a transgene can reduce LDLR protein levels in the liver and consequently elevate cholesterol in mice (Lagace et al., 2006; Greffhorst et al., 2008; Schmidt et al., 2008).

Evidence also points to the ability of intracellular PCSK9 to degrade LDLR. Although inhibition of PCSK9 using a neutralizing antibody can reduce LDL cholesterol in nonhuman primates by 80%, a level that might be expected for a complete or near-complete inhibition of PCSK9 function, the same antibody, with 160 m Binding affinity to mouse PCSK9, only showed partial reduction of total cholesterol in wild-type mice (36%) compared with the reported 52% for the PCSK9(-/-) mice (Rashid et al., 2005; Chan et al., 2009). In vitro, an antibody-mediated reversal of the PCSK9 effect on LDL uptake was lower in cultured cells when PCSK9 was overexpressed in the cells rather than being added exogenously as a purified protein (Duff et al., 2009). Overexpression of PCSK9 in HepG2 cells has been shown to increase the degradation of the precursor LDLR in addition to mature LDLR (Maxwell et al., 2005). Moreover, transfection of a PCSK9-expressing construct can reduce LDLR levels in PCSK9-expressing cells, but not in surrounding nontransfected cells (Poirier et al., 2009). Finally, two PCSK9 mutants, S127R and D129G, were reported to have reduced or apparently abolished secretion but they are associated with familial hypercholesterolemia, strongly implying that intracellular PCSK9 can still participate in LDLR degradation (Benjannet et al., 2006; Cameron et al., 2006; Homer et al., 2008). In our experiments, the degree of reduction (46%) in J10-treated mice was very similar to that observed in PCSK9(-/-) mice in our hands (45%) and reported by others (52%) (Rashid et al., 2005), as well as that reported for antisense knockdown of PCSK9 (50%) (Graham et al., 2007; Frank-Kamenetsky et al., 2008; Gupta et al., 2010). We show that this reduction in serum cholesterol is through PCSK9 inhibition and, consequently, LDLR up-regulation, because J10 does not have any detectable effect in mice lacking LDLR, and LDLR protein level is increased in J10-treated animals compared with controls. Because we are not aware of evidence suggesting antibodies can interfere with the intracellular function of a protein, our results indicate that neutralizing circulating PCSK9 via a monoclonal antibody can remove most PCSK9 function under normal physiological conditions and strongly suggest that the vast majority, if not all, of PCSK9 mediated cholesterol modulation effect is carried out via secreted PCSK9, at least in the mouse.

We show that PCSK9 antagonism can effectively lower LDL-C levels without significantly affecting HDL-C or triglyceride levels in both normal and hypercholesterolemic monkeys. Although several means of PCSK9 antagonism have been reported to reduce LDL-C levels by 50 to 80% in normal monkeys (Frank-Kamenetsky et al., 2008; Chan et al., 2009; Ni et al., 2011), we show for the first time that PCSK9 antagonism is effective in a hypercholesterolemic monkey model caused by a long-term high-fat diet. An antisense oligo-specific to mouse PCSK9 has been reported to reduce serum cholesterol by 53% in mice fed a Western diet (60% lard) (Graham et al., 2007). However, our anti-PCSK9 monoclonal antibody had no significant effect on mice fed HF or HF/HC diets, nor did we see a significant difference in PCSK9(-/-) fed a HF/HC diet. Differences in diet, experimental conditions, and housing conditions may be responsible for this discrepancy. In our case, the HF diet decreased serum PCSK9 levels by 64%, possibly partly explaining the lack of effect of further PCSK9 inhibition or knockout in diet-induced hypercholesterolemic mice. Our data are also consistent with reports that dietary cholesterol inhibits PCSK9 expression in rat liver (Persson et al., 2009). Unlike rodents where hypercholesterolemia caused by a diet high in cholesterol suppresses PCSK9 levels, studies have found that hypercholesterolemic humans have elevated PCSK9 levels (Lakoski et al., 2009; Welder et al., 2010). It is likely that the unresponsiveness to diet-induced hypercholesterolemia is a property of rodents but not primates, because we have shown that both the degree and duration of LDL-C reduction are similar in normal and hypercholesteremic monkeys treated with the same dose of the antagonistic PCSK9 antibody J16.

Finally, we show that PCSK9 antagonism results in effective LDL-C in statin-treated hyperlipidemic monkeys. It is noteworthy that one of the most potent statins commercially available, rosuvastatin, had no effect on plasma cholesterol levels in these monkeys with a daily dose of 20 mg/kg. Treatment with a high dose (50 mg/kg/day) of simvastatin caused a 43% reduction in LDL-C and a 32% reduction in HDL-C. Addition of a single administration of J16 on top of simv-
statin treatment caused an additional 65% lowering of LDL-C with no significant effect on HDL-C. The percentage of LDL-C lowering was similar to the 64% lowering observed with J16 monotherapy in these monkeys, demonstrating a potential additive effect of treatment with a PCSK9 monoclonal antibody paired with that of statin. It is noteworthy that we have not observed any significant changes related to statin or J16 treatment in body weight, clinical signs, and liver or kidney function throughout the study. Our results provide further confidence that PCSK9 antagonism, particularly anti-PCSK9 monoclonal antibodies, can be a promising new lipid-modifying therapy that warrants further clinical testing.

Acknowledgments

We thank Shirish Shenolikar, Julie Hawkins, Yuli Wang, Kieran Geoghegan, and Xiayang Qu for protocols, recombinant human PCSK9 protein, and discussions; Leila Boustanian for assistance with construct generation, manuscript editing, and discussions; Kathy Tsui and Zea Melton for assistance with mouse hybridoma work; Terri Martin for assistance in expressing J16; Daniel Malashock, Alanna Pinkerton, and Kevin Lindquist for assistance with biosensor work; and Duc Tien, Joyce Chou, Ariel Pios, German Vergara, Iketem Meriwerther, Ronald Ong, Teresa Radcliffe, and Gustavo Ruiz for assistance with animal work.

Authorship Contributions

Participated in research design: Liang, Strop, Abidche, Lin, Rajal, Pons, and Shelton.
Conducted experiments: Liang, Chaparro-Riggers, Strop, Geng, Sutton, Tsai, Dalley, Yu, and Wu.
Contributed new reagents or analytic tools: Abidche, Chin, and Lee.
Performed data analysis: Liang, Strop, Abidche, and Rossi.

References

Rau, Pons, and Shelton.


Address correspondence to: Hong Liang, Rinat Laboratories, Pfizer Inc., 230 East Grand Avenue, South San Francisco, CA 94080. E-mail: hong.liang@pfizer.com